JOURNAL OF BACTERIOLOGY
Vol. 88, No. 5, p. 1403–1415 November, 1964

EFFECT OF SUGARS AND OTHER CARBON COMPOUNDS ON GERMINATION AND POSTGERMINATIVE DEVELOPMENT OF *BACILLUS MEGATERIUM* SPORES[1]

MILDRED T. HYATT AND HILLEL S. LEVINSON
Pioneering Research Division, U.S. Army Natick Laboratories, Natick, Massachusetts

Received for publication 20 July 1964

ABSTRACT

HYATT, MILDRED T. (Pioneering Research Division. U.S. Army Natick Laboratories, Natick, Mass.), AND HILLEL S. LEVINSON. Effect of sugars and other carbon compounds on germination and postgerminative development of *Bacillus megaterium* spores. J. Bacteriol. **88**:1403–1415. 1964.—A total of 77 carbon-containing compounds were tested for their ability to support germination and postgerminative development of *Bacillus megaterium* spores. The only effective germination agents were certain of the hexose sugars and their derivatives. With unheated spores, only D-glucose, D-mannose, 2-deoxy-D-glucose, D-glucosamine, and *N*-acetyl-D-glucosamine (all at 25 mM) supported appreciable germination (ca. 25%). Heat-shock at 60 C for 10 min increased germination and decreased the concentration of sugar required for germination, so that these compounds, at 2.5 mM, supported 40 to 60% germination. Higher concentrations (25 mM) of other compounds, D-fructose, L-sorbose, D-allose, D-altrose, 2-hydroxyethyl-D-glucose, and β-methyl-D-glucoside, were required for appreciable germination of heated spores. Glucose or mannose contamination accounted for the germination apparently induced by certain other sugars. Ionic contamination did not appear to contribute to the germination induced by D-glucose, D-fructose, 2-deoxy-D-glucose, or L-sorbose. There was no clear-cut evidence for a multiplicity of metabolic pathways in the triggering of *B. megaterium* spore germination by various sugars. Postgerminative development of germinated spores was supported by a wider variety of carbon compounds, including some pentoses and hexoses, many oligosaccharides, sugar derivatives, some alcohols, and some of the tricarboxylic acid cycle intermediates. Compounds effective for germination were not necessarily utilizable for growth, and vice versa. Oxygen consumption rates reflected the progress and extent of postgerminative development on the various carbon compounds. Utilization of glucose during postgerminative development was followed, and the concentration requirements were determined.

Bacillus megaterium spore germination is triggered by a wide variety of compounds of quite diverse nature, including glucose, L-alanine, or KNO_3 (Hills, 1950; Powell, 1951; Hyatt and Levinson, 1962). It is evident, therefore, that neither a carbon nor nitrogen source is specifically required for germination. Postgerminative development of the germinated spore into a dividing vegetative cell, however, requires sources of phosphorus, sulfur, and nitrogen, as well as a metabolizable carbon source, such as glucose (Hyatt and Levinson, 1957, 1959; Levinson and Hyatt, 1962).

Levinson and Hyatt (1962) showed that nitrogenous compounds supporting germination do not necessarily support postgerminative development, and also (Hyatt and Levinson, 1962) found that germination may occur via diverse metabolic pathways. The present report extends the observations with nitrogenous compounds to carbon compounds, 77 compounds having been examined for their ability to support germination and postgerminative development. Some of these have already been listed as germination agents for various species of *Bacillus*, but we know of no other investigation of such a large number of carbon compounds in postgerminative development. By variation of germination conditions, we investigated the possibility that different sugars trigger germination through different metabolic pathways. On the basis of our experiments on oxygen consumption rates during postgerminative development in various carbon compounds, we discuss, and to some extent confirm, the observations of Hachisuka et al. (1956) that oxidative systems develop in a stepwise

[1] A portion of this paper was presented at the Annual Meeting of the American Society for Microbiology in Washington, D.C., 3–7 May 1964.

manner as the germinated spore develops into a vegetative cell.

MATERIALS AND METHODS

The same pool of *B. megaterium* (QM B1551) spores was used as previously described (Levinson and Hyatt, 1962). Briefly, the spores were grown on liver broth (0.8% Liver Fraction "B," The Wilson Laboratories, Chicago, Ill.), buffered initially at pH 6.5 with 10 mM potassium phosphate, harvested and washed by repeated centrifugation at 4 C, and then lyophilized. Spores at a final concentration of 1 mg (5×10^8 spores) per ml were used, either unheated or heated in water suspensions at 60 C for 10 min, unless other heating conditions are indicated.

Compounds of the highest commercially available reagent grade were used throughout. D-Glucose, D-fructose, and L-sorbose (all from Pfanstiehl Laboratories, Inc., Waukegan, Ill.); D-mannose (Fisher Scientific Co., Pittsburgh, Pa.); and 2-deoxy-D-glucose (Calbiochem) were studied in detail as germination agents. These sugars and all other compounds active in inducing germination were tested for trace sugar contamination. Glucose contamination was estimated by an enzymatic method with the use of glucose oxidase (Glucostat; Worthington Biochemical Corp., Freehold, N.J.); other reducing sugars were developed on paper chromatograms with *n*-butanol–*n*-propanol–ethanol–water (2:3:1:3), and were sprayed with alkaline $AgNO_3$. Certain compounds were freed from glucose by incubating them for 1 hr with glucose oxidase [1 ml of 250 mM sugar solution and 1 ml of glucose oxidase (12.5 mg of Glucostat reagent)]. Glucose-free α- and β-methyl-D-glucosides were also prepared by passage through a mixed bed resin (Amberlite, MB 1), the anion component of which removes traces of reducing sugars (Phillips and Pollard, 1953). Essentially glucose-free galactose was obtained from the Sigma Chemical Co., St. Louis, Mo., and pure gentiobiose from the Pillsbury Co., Minneapolis, Minn. The "glucose-free" compounds were compared with the reagent compounds in germination-inducing abilities.

In experiments with "deionized" germinants, deionized water (resistivity at least 1.5×10^6 ohm-cm) prepared by passage through Amberlite MB 1 and glassware washed with it were used throughout; 10% solutions of the sugars were deionized with the resin (50 ml of solution to 10 g of resin). Treatment with the resin increased the resistivities of the sugar solutions from ca. 10^5 to 8×10^5 ohm-cm, and ca. 70% of each sugar was recovered. Spores, washed three times in deionized water, were germinated in unbuffered water solutions (pH, ca. 7.0) of the deionized germinants.

For germination studies, 3.0 ml of spore suspensions plus carbon compounds in phosphate buffer (50 mM, pH 7.0) were shaken in 50-ml Erlenmeyer flasks in a water bath at 30 C. Slides were prepared for microscopic examination at intervals to 2 hr, and germination was determined by the percentage of spores staining with dilute methylene blue.

For study of the promotion of postgerminative development by various carbon compounds, heated spores were germinated by incubation at 30 C in L-alanine (25 mM), potassium phosphate (50 mM), K_2SO_4 (1 mM), and glucose (0.25 mM). This medium meets the nitrogen, phosphate, and sulfur requirements for postgerminative development (Hyatt and Levinson, 1957, 1959; Levinson and Hyatt, 1962). The small amount of glucose (0.25 mM) was insufficient to support postgerminative development, but did promote rapid and synchronous germination in L-alanine (80% germination in 15 min); by 30 min, when various carbon sources (25 mM) were added, all of the original glucose had been utilized by the germinated spores and had disappeared from the medium (Levinson and Hyatt, *in press*). Postgerminative development was followed over a 6-hr period. Experiments on the effects of carbon compounds on postgerminative development were done in 50-ml Erlenmeyer flasks, but, when oxygen consumption was being measured, standard Warburg techniques were employed. Glucose utilization was estimated by assay (dinitrosalicylic acid method of Sumner, 1925) of glucose remaining in supernatant fluids from Warburg flasks at intervals during postgerminative development.

RESULTS

Germination

Effect of various carbon compounds. A total of 77 carbon compounds were tested for their ability to support germination of unheated or heated spores (Table 1). The only effective compounds

were certain of the hexose sugars or their derivatives. With unheated spores, only D-glucose, D-mannose, 2-deoxy-D-glucose, D-glucosamine, and *N*-acetyl-D-glucosamine (all at 25 mM) supported appreciable germination (ca. 25%). Heating of spores at 60 C for 10 min not only increased the percentage of germination induced by these sugars and allowed a lower concentration (2.5 mM) to be effective, but also permitted low levels of germination (10 to 18%) in additional compounds (2.5 mM D-fructose, L-sorbose, D-galactose, 2-hydroxyethyl-D-glucose, and α- and β-methyl-D-glucosides). Other sugars (D-erythrose, D-allose, D-altrose, cellotriose, cellotetraose, gentiobiose, maltose, sophorose, 3-*O*-methyl-D-glucose, and α-methyl-D-mannoside) were ineffective at 2.5 mM but, at 25 mM, did support germination of heated spores. However, since D-glucose was an effective germination agent for heated spores at very low concentration levels (Hyatt and Levinson, 1962), we investigated the possibility that trace contamination with glucose or other sugars might be contributing to the germination reported for some of these compounds. All of the germination with maltose (13%) and with 3-*O*-methyl-D-glucose (12%) could be accounted for by glucose contamination; all of the germination with α-methyl-D-mannoside (17%) could be accounted for by mannose contamination. With certain other sugars, glucose contamination was insufficient to account for all of the reported germination, but, in these cases, traces of glucose might be acting synergistically with these compounds in promoting germination. The drastic reduction in germination in D-erythrose, D-galactose, cellotriose, cellotetraose, gentiobiose, sophorose, and α-methyl-D-glucoside, when freed from glucose contamination by treatment with glucose oxidase (Table 1) made it doubtful that these sugars were truly germination agents. Germination in β-methyl-D-glucoside was not reduced by treatment with glucose oxidase; therefore, in spite of this sugar's 1.1% glucose contamination, it was probably a true inducer of germination. Furthermore, β-methyl-D-glucoside freed from glucose by passage through a mixed bed resin retained most of its germination-inducing capacities (37% germination at 25 mM, with heated spores), but α-methyl-D-glucoside, so treated, did not (only 6% germination). Commercially available, essentially glucose-free galactose and gentiobiose were also completely devoid of germination-inducing capacities. The compounds which we do consider to be active germination agents for *B. megaterium* spores are starred (*) in Table 1.

It was claimed that ions contaminating glucose are the primary germination agents for glucose-induced germination of *B. megaterium* spores, heated at 60 C for 1 hr (Rode and Foster, 1962*a*). Indeed, it is possible that germination induced by D-glucosamine may be partly due to Cl^-, because this compound was used as the HCl. However, we found (data not shown) that deionization of D-glucose, D-fructose, L-sorbose, or 2-deoxy-D-glucose did not result in a reduction in the germination-inducing abilities of these sugars, either with unheated or heated spores (60 C for 10 min). At low substrate levels, however, the contribution of ions to D-mannose–induced germination may be significant. At 0.05 mM, deionized D-mannose induced only 17% germination of heated spores, but reagent D-mannose induced 38% germination. [Germination in experiments on the effect of deionizing carbon compounds was higher than in other experiments. Several possibilities were explored (e.g., use of deionized water might be contributing amines; excessive centrifugation of spores during washing). However, it was determined that the absence of the phosphate buffer was responsible for the higher germination, in agreement with our previous observations that high phosphate concentrations are inhibitory to spore germination (Levinson and Sevag, 1953).]

The rate of germination of heated spores incubated with various selected sugars is shown in Fig. 1. There was no appreciable lag (at 15 min) in inception of germination with any of the compounds tested. With D-glucose, the full germination potential (no further increase in percentage germination with increased incubation time) was reached within 30 min. In D-fructose, D-mannose, L-sorbose, and 2-deoxy-D-glucose, germination was more gradual, but full germination potential was attained within 2 hr in all cases.

Conditions affecting germination. Of the sugars listed as effective germination agents in Table 1, three (D-glucose, D-mannose, and 2-deoxy-D-glucose) that supported germination of both unheated and heated spores and two (D-fructose and L-sorbose) that were effective only with heated spores were selected for more detailed

TABLE 1. *Germination and postgerminative development of Bacillus megaterium spores in the presence of carbon compounds*[a]

Carbon source	Contamination of sugars determined by		Germination[b]				Postgerminative development[c]	
	Glucostat (glucose)	Paper chromatography	Reagent compounds			Glucose-free compounds[d] (heated spores)	Elongation	Cell division
			Unheated spores	Heated spores				
			25 mM	2.5 mM	25 mM	25 mM		
	%		%	%	%	%	%	%
Sugars								
Monosaccharides								
D-Erythrose	1.1		8	8	44	4	0	0
D-Ribose			0	1	1		100	78
D-Xylose			0	1	3		81	62
D-Allose*	0		2	3	33	31	0	0
D-Altrose*		Pure	1	3	24			
D-Fructose*	0	Pure	3	17	24	25	99	80
D-Galactose	3.5		9	18	48	1	70	50
D-Glucose*		Pure	28	60	80		99	81
D-Gulose·$CaCl_2$[e]			3	5	10			
D-Mannose*	0	Pure	30	64	82	76	0	0
L-Sorbose*	0	Pure	4	14	27	28	0	0
Oligosaccharides								
Cellobiose			1	1	2		65	30
Cellotriose	0.13		1	1	31	4	50	28
Cellotetraose	0.10		1	1	50	4	35	9
Gentiobiose	1.0		0	2	69	3	99	75
Lactose			0	1	1		99	45
Maltose	0.4		2	2	13		97	65
Melezitose			1	2	5		27	0
Melibiose			1	3	5		97	71
Raffinose			0	1	1		92	52
Sophorose	0.55		1	4	48	6	0	0
Sucrose			1	2	5		90	62
Trehalose			1	1	1		99	75
Turanose			1	1	2		98	58
Alcohols								
Glycerol			1	1	1		100	72
Inositol			1	1	1		47	2
Mannitol			0	1	1		80	60
Miscellaneous								
2-Deoxy-D-glucose*		Pure	24	50	73	71	0	0
D-Galactonate (K)			0	1	1		81	22
D-Glucosamine·HCl*[e]	0.17		29	60	82	70	100	79
N-Acetyl-D-glucosamine*	0		24	40	78	79	99	83
D-Gluconate (Ca)			1	1	1		92	79
D-Glucuronate (K)			0	0	1		36	1
2-Hydroxyethyl-D-glucose*	0.15		10	15	66	50	0	0
2-D-Ketogluconate (Ca)			1	3	3		98	64
3-*O*-methyl-D-glucose	0.52		2	4	12		0	0
α-Methyl-D-glucoside	0.3		2	10	31	6	34	2
β-Methyl-D-glucoside*	1.1		8	11	53	50	95	64
α-Methyl-D-mannoside	0	Mannose (0.4%)	1	1	17	18	0	0

TABLE 1.—*Continued*

Carbon source	Contamination of sugars determined by		Germination[b]				Postgerminative development[c]	
	Glucostat (glucose)	Paper chromatography	Reagent compounds			Glucose-free compounds[d] (heated spores)	Elongation	Cell division
			Unheated spores	Heated spores				
			25 mM	2.5 mM	25 mM	25 mM		
	%		%	%	%	%	%	%
Tricarboxylic acid cycle compounds								
Citrate (Na)			1	1	1		39	0
Fumarate (K)			0	1	1		99	81
Malate (K)			1	1	1		90	60
Succinate (Na)			0	1	1		91	70
Miscellaneous carbon compounds								
L-Aspartate (K)			0	1	1		98	71
L-Glutamate (K)			1	1	6		95	44

[a] The following compounds are inactive, i.e., they support neither germination nor postgerminative development. (i) Sugars: monosaccharides: D-glyceraldehyde, dihydroxyacetone, D-arabinose, L-arabinose, D-lyxose, L-fucose, L-glucose, L-mannose, L-rhamnose, and D-talose; phosphorylated sugars (with or without 0.4 mM $MnSO_4$): fructose-1,6-diphosphate (Na), fructose-6-phosphate (Na), glucose-1-phosphate (K), glucose-6-phosphate (Na), glycerophosphate (K), 6-phosphogluconate (Ba), and ribose-5-phosphate (Na); alcohols: adonitol, dulcitol, erythritol, and sorbitol; miscellaneous: L-ascorbate (K), L-gulonate (K), D-mannonate (K), and α-methyl-D-xyloside. (ii) Tricarboxylic acid cycle compounds: acetate (Na), α-ketoglutarate (K), lactate (K), and pyruvate (Na). (iii) Miscellaneous carbon compounds: carbonate (K), ethyl alcohol, and formaldehyde.

[b] Germination was determined after 2 hr of incubation in phosphate buffer (50 mM, pH 7.0), plus carbon sources. No germination in phosphate alone.

[c] For postgerminative development studies, heated spores were germinated (80%) in a medium containing L-alanine (25 mM), D-glucose (0.25 mM), K_2SO_4 (1 mM), and phosphate buffer (50 mM) for 30 min, before the addition of carbon sources (25 mM). Numbers represent percentages of germinated spores which elongate or divide in 6 hr.

[d] Sugars freed from glucose contamination by incubation with glucose oxidase for 1 hr.

[e] Cl^- may be contributing to germination.

* We consider these compounds to be active germination agents.

study of the effects of varying conditions of germination.

Concentration requirements:—The compounds which supported germination of unheated spores (D-glucose, D-mannose, and 2-deoxy-D-glucose) were more effective germination agents for heated spores than were D-fructose and L-sorbose (Fig. 2). For 50% germination, 1.0 mM D-mannose, 2.0 mM D-glucose, and 4.0 mM 2-deoxy-D-glucose sufficed, but much higher concentrations were required for the other compounds (70 mM L-sorbose and 250 mM D-fructose). Germination of heated spores in D-glucose, D-mannose, and 2-deoxy-D-glucose reached nearly maximal levels at 25 mM, but germination in the other two compounds continued to increase, with concentrations as high as 250 mM. In subsequent experiments, concentrations of these sugars were adjusted to give roughly equivalent germination.

Effect of pH:—Heated spores were incubated for 2 hr in phosphate buffer at pH levels from 5 to 8 (Fig. 3) in the presence of the five germinants (D-glucose, D-mannose, and 2-deoxy-D-glucose at 2.5 mM; the other compounds at 50 mM). Germination in D-glucose occurred over a wide pH range, but was somewhat lower at pH 8. Germination in D-mannose and in 2-deoxy-D-glucose was optimal at pH 7, and in L-sorbose at pH 6.

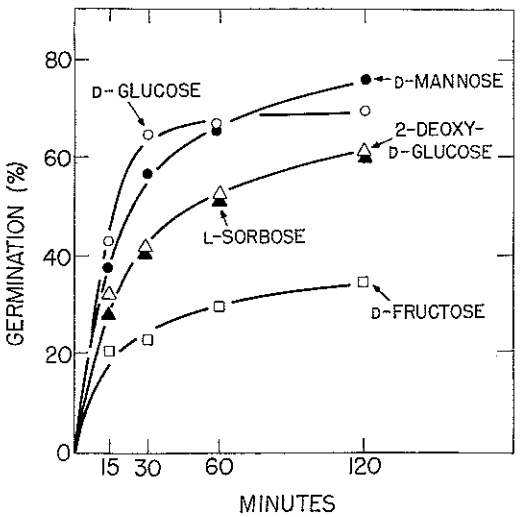

FIG. 1. *Germination of spores of Bacillus megaterium in various sugars. Germination was plotted as a function of time. Aqueous suspensions of spores were heated at 60 C for 10 min before incubation at 30 C in phosphate buffer (50 mM, pH 7.0) plus indicated sugars. D-Glucose, D-mannose, and 2-deoxy-D-glucose were used at 10 mM; D-fructose and L-sorbose, at 100 mM.*

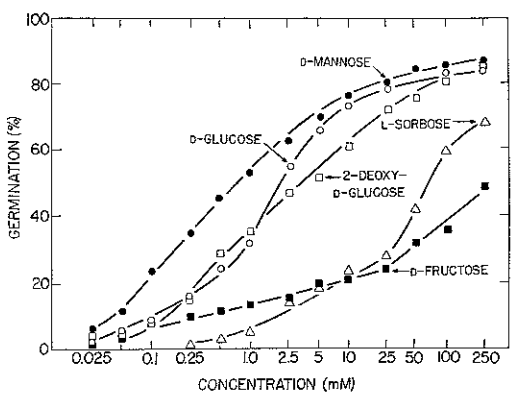

FIG. 2. *Effect of concentration of various sugars on the germination of spores of Bacillus megaterium. Germination was determined at 2 hr. Aqueous suspensions of spores were heated at 60 C for 10 min before incubation at 30 C in phosphate buffer (50 mM, pH 7.0) plus indicated sugar.*

Germination in D-fructose reached higher levels on the acid side of neutrality.

Temperature of heat activation and of incubation:—Spore suspensions were heated for 10 min at temperatures between 40 and 90 C. The critical temperature for heat activation of germination appeared to lie between 50 and 52.5 C (Fig. 4; data plotted only for D-glucose, but similar responses were obtained with the other four sugars). Heat treatment at 80 C gave the highest total germination with all five sugars. Spores, heated at 90 C, showed a lag in the initiation of germination, but the total germination (in 2 hr) was the same as for spores heated at 57.5 C.

The early rate and extent of germination depended on the incubation temperature (Fig. 5; data plotted only for D-glucose, but similar responses were obtained with other sugars). The extent of germination was virtually the same after incubation for 120 min at 20, 30, and 40 C, but the initial rate of germination was maximal at 30 C. Germination after 120 min at 13.5 and at 45 C were both about 60% that at 30 C, but at 13.5 C there was a long lag in inception of germination, only 2% of the spores germinating in 15 min. Germination at 50 C was quite slow, and in 120 min only 10% of the spores had germinated.

Chemical inhibition of germination:—Germination induced by the five sugars was not significantly differentially inhibited by various enzyme inhibitors and chelating agents. Depending on the sugar used, there was 34 to 54% inhibition of germination with dipicolinic acid (25 mM), 4 to

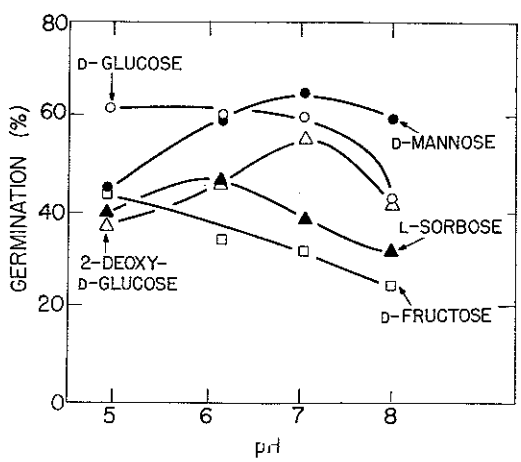

FIG. 3. *Effect of pH on germination of spores of Bacillus megaterium in various sugars. Germination was determined at 2 hr. Spores were heated at 60 C for 10 min before incubation at 30 C at the indicated pH levels in phosphate buffers (50 mM) plus sugars. D-Glucose, D-mannose, and 2-deoxy-D-glucose were used at 2.5 mM; D-fructose and L-sorbose, at 50 mM.*

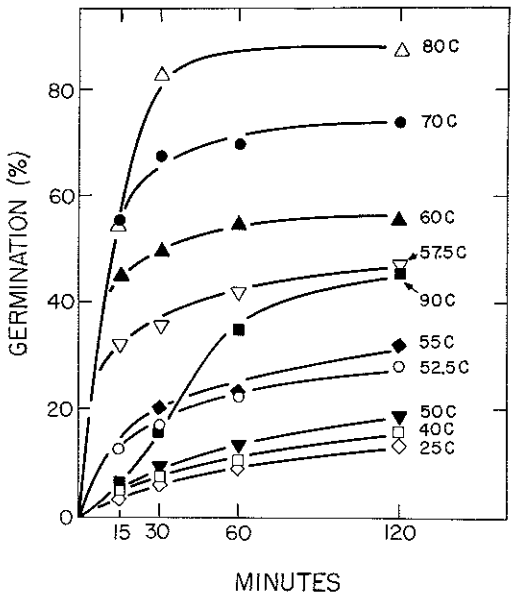

FIG. 4. *Effect of temperature of "heat shock" on the glucose-induced germination of spores of Bacillus megaterium. Similar heat-activation responses were obtained with* D*-mannose, 2-deoxy-*D*-glucose,* D*-fructose, and* L*-sorbose. Germination was plotted as a function of time. Aqueous suspensions of spores were heated for 10 min at the indicated temperatures before incubation at 30 C in phosphate buffer (50 mM, pH 7.0) and glucose (2.5 mM).*

16% with 8-hydroxyquinoline (5 mM), 0 to 12% with ICH_2COOK (10 mM), 21 to 35% with NaN_3 (10 mM), and 20 to 35% inhibition with NaF (100 mM). Germination in all five sugars was completely inhibited by 1 mM $HgCl_2$. There was no inhibition with 0.5 mM $HgCl_2$; with 1 mM *p*-chloromercuribenzoate, Atebrin, isonicotinic acid hydrazide, 2,3-dimercaptopropanol, KCN, sodium diethyldithiocarbamate; or with 10 mM 2,4-dinitrophenol, ethylenediaminetetraacetic acid, or $NaAsO_2$.

Combinations of germination agents:—Heated spores and various germinants in concentrations sufficient to support ca. 10 to 15% germination when used singly were incubated in combinations. Germination in 0.25 mM L-alanine was stimulated equally by addition of all five of the selected sugars (ca. 100% increase over sum of germination in germinant plus additive). Germination in 0.25 mM D-glucose was not stimulated by addition of D-mannose and 2-deoxy-D-glucose, but was stimulated by addition of D-fructose (56%) and, to a lesser degree, by L-sorbose (19%). Germination in 1.0 mM D-fructose was not stimulated by addition of L-sorbose, but was somewhat stimulated by the addition of the other compounds (16 to 39%).

Sporulation medium:—Spore germination characteristics (including heat-activation requirements) of *B. megaterium* spores are determined both by the composition of the sporulation medium and by the germination agent (Levinson and Hyatt, 1964). In current studies, spores produced on several media were tested for germination on the various sugars (Table 2). Spores produced on the "basal" medium (Liver Fraction "B", 0.5%; plus KH_2PO_4, 10 mM) did not germinate on any of the sugars unless they were heated. The heat-activation requirement of "$CaCl_2$ spores" for germination on D-glucose, D-mannose, and 2-deoxy-D-glucose was reduced markedly; that for D-fructose–induced germination was reduced less. "Glutamate spores" had reduced heat-activation requirements for germination on all of the sugars,

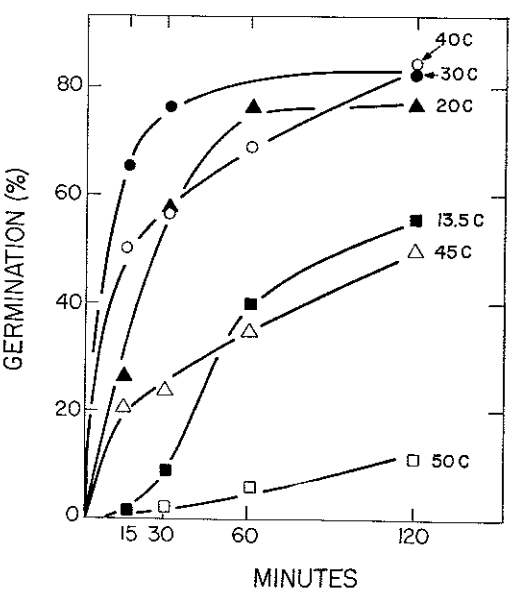

FIG. 5. *Effect of temperature of incubation on the germination of spores of Bacillus megaterium in glucose. Similar responses were obtained with* D*-mannose, 2-deoxy-*D*-glucose,* D*-fructose, and* L*-sorbose. Germination was plotted as a function of time. Aqueous suspensions of spores were heated at 60 C for 10 min before incubation at the indicated temperatures in phosphate buffer (50 mM, pH 7.0) plus glucose (25 mM).*

TABLE 2. *Effect of sporulation medium on the heat-activation requirement for germination of Bacillus megaterium spores in various sugars*

Germinant		Heat activation (60 C, 10 min)	Germination[a] of spores produced on various media[b]				
Sugar	Concn		Liver "B", 0.5% ("basal")	"Basal" + $CaCl_2$ (0.5 mM)	"Basal" + glutamate (12.5 mM)	Liver "B,"[c] 0.8%	Synthetic medium
	mM		%	%	%	%	%
D-Glucose	10	−	3	27	50	21	52
		+	75	89	95	78	94
D-Mannose	10	−	4	38	73	19	55
		+	84	93	97	78	98
2-Deoxy-D-glucose	10	−	1	22	36	12	56
		+	70	93	80	75	98
D-Fructose	50	−	3	12	27	6	62
		+	52	82	86	43	94
L-Sorbose	50	−	1	4	16	5	43
		+	65	79	90	55	99

[a] Percentage germination of unheated (−) or of heated (+) spores was determined after incubation (2 hr at 30 C) with various sugars in phosphate buffer (50 mM, pH 7.0).

[b] All sporulation media contained 10 mM phosphate (Levinson and Hyatt, 1964).

[c] Medium containing 0.8% Liver Fraction "B" was used for production of the spores used through the remainder of the study.

[d] Synthetic medium (Levinson and Hyatt, 1964) included glutamate (10 mM) and $CaCl_2$ (0.5 mM).

but this was most marked for germination on D-glucose and D-mannose. Germination of both heated "$CaCl_2$ spores" and "glutamate spores" was increased over that of "basal spores" so that, in the concentrations used, all of the germinants were equally effective. The spores used in most of our experiments were grown on the "basal" medium supplemented so as to contain 0.8% Liver Fraction "B." These spores had reduced heat-activation requirements for germination induced by D-glucose, D-mannose, and 2-deoxy-D-glucose, probably because of the increased Ca^{++} content of the additional liver (Levinson and Hyatt, 1964). Reduction of the requirement for heat activation for germination of spores produced on the synthetic medium may be attributed to the $CaCl_2$ and L-glutamate content of that medium.

Postgerminative development

Effect of various carbon compounds. Promotion of spore germination by a carbon compound does not necessarily imply utilization of the compound in postgerminative development (Table 1). Indeed, although D-glucose supports full postgerminative development, D-mannose and 2-deoxy-D-glucose, which are fully as effective as glucose in inducing germination of both unheated and heated spores, cannot be used as carbon sources for growth. Conversely, many compounds, completely ineffective as germination agents, are excellent carbon sources for postgerminative development. Many other compounds support neither germination nor growth. Because much higher concentrations of glucose are required for postgerminative development than for germination, trace glucose contamination of carbon sources need not be considered here.

Postgerminative development of ionically germinated spores. The question of whether ionically germinated spores (Rode and Foster, 1962*a*) were capable of undergoing postgerminative development was investigated. Spores, heated at 60 C for 30 min, were ionically germinated in 10 mM KCl, in a medium suitable for postgerminative development (25 mM trehalose, as a carbon source; 25 mM $(NH_4)_2SO_4$; 50 mM phosphate; pH 7.0). Trehalose alone did not support germination, nor did it, in combination with KCl, increase the 40% germi-

nation obtained with KCl alone. After 6 hr in this medium, 70% of the germinated spores had elongated and 60% had divided. This postgerminative development compared favorably with that of spores germinated physiologically in L-alanine, with trehalose as the carbon source.

Oxygen consumption in the presence of various carbon sources. The morphological stages of spore germination and subsequent growth coincided with changes in respiratory activity, the respiratory rate curves being characterized by linear increases in rate corresponding to the phases of germination, swelling, elongation, and cell division (Levinson and Hyatt, 1956). Heated spores, incubated after germination in a medium suitable for postgerminative development, had different oxygen uptake rates with different carbon sources (Fig. 6). In general, these curves reflected the progress and extent of postgerminative development on the various carbon compounds. D-Glucose [as well as malate, maltose, sucrose, trehalose, and gentiobiose (data not shown)] promoted the most rapid and complete development. Development of germinated spores in gluconate (as well as in D-fructose, glycerol, mannitol, *N*-acetyl-D-glucosamine, 2-D-ketogluconate and fumarate) was only slightly delayed. In D-ribose (and also with D-xylose, lactose, melibiose, turanose, raffinose, D-galactose, and L-aspartate), oxygen uptake rates associated with cell division did not start until 190 min (vs. 130 min with glucose). With D-glucosamine (and also with L-glutamate, β-methyl-D-glucoside, cellobiose, and succinate), cell division did not start until 230 min. With α-methyl-D-glucoside (as well as melezitose, inositol, glucuronate, and citrate), the germinated spores swelled, emerged, and elongated, but only a few cells started to divide within 6 hr, and this was reflected in delayed rate changes in oxygen consumption. Oxygen consumption rates in carbon compounds such as D-mannose (also 2-deoxy-D-glucose, L-rhamnose, and L-arabinose), which did not support postgerminative development, failed to increase over the rate of the germinated spores incubated in a medium devoid of an exogenous carbon source.

Chemical inhibition. Postgerminative development in 25 mM D-glucose, gluconate, or fumarate was inhibited by 1 mM Atebrin and by 10 mM KCN, but not by 10 mM NaF. With NaF present, there was a 10-min delay in attainment of normal oxygen consumption rates at all stages of development in D-glucose, and a 30-min delay in gluconate and fumarate. However, by 6 hr, spores incubated with NaF had attained normal development.

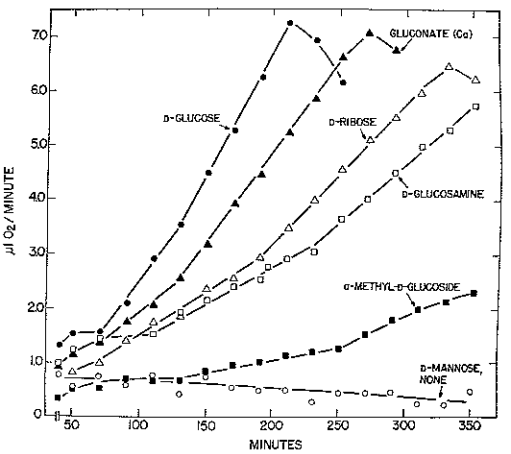

FIG. 6. *Oxygen consumption rates during the postgerminative development of Bacillus megaterium spores incubated with various carbon compounds. Spores were germinated for 30 min (80% germination) in L-alanine (25 mM), glucose (0.25 mM), K_2SO_4 (1 mM), and phosphate buffer (50 mM, pH 7.0) before addition of carbon compounds (25 mM).*

Glucose concentration requirements and utilization. Heated spores, germinated in a medium containing only 0.25 mM D-glucose, but otherwise suitable for postgerminative development, were incubated with an additional 0 to 50 mM glucose. In the absence of additional D-glucose, no postgerminative development occurred, and the oxygen uptake rate did not increase over that of the germinated spores (Fig. 7); 1 mM D-glucose was insufficient to support growth and concomitant increases in rate of oxygen consumption. With 5 mM D-glucose, swelling, emergence, and elongation occurred, accompanied by increasing rates of oxygen consumption. However, 5 mM D-glucose did not support cell division or a continued high rate of oxygen consumption, and at 150 min there was a decline in the oxygen uptake rate. With higher concentrations of D-glucose, there was a change in the slope of oxygen uptake rate curves at 130 min, marking the beginning of cell division. In 10 mM D-glucose, about 60% of the germinated spores had started to divide when the oxygen consumption rate fell at 190 min; in 15 mM D-glucose, about 70% of the spores had divided, and the oxygen uptake rate declined at 230 min.

With concentrations of D-glucose of 25 to 50 mM, 80 to 90% of the spores underwent two cell divisions, but the rate of oxygen consumption also declined at 230 min. The interesting rise in oxygen uptake rate which occurs with 10 mM D-glucose, after exhaustion of D-glucose (see below), is currently under investigation.

The utilization of D-glucose, at an initial concentration of 25 mM (6.75 mg of glucose in 1.5 ml of reaction mixture), was determined at intervals during incubation of heated spores undergoing postgerminative development (Fig. 7, broken line). Very little D-glucose is utilized during the swelling stages after germination. Some D-glucose is utilized during elongation, but the major disappearance of D-glucose from the medium takes place during cell division, about 50% (3.5 mg) of the 25 mM D-glucose disappearing by the completion of one cell division (230 min). It is not surprising, therefore, that neither 5 mM (1.35 mg) nor 10 mM (2.7 mg) D-glucose was sufficient to allow spores to complete their development, the drops in oxygen consumption rates at 150 and 190 min reflecting exhaustion of D-glucose. On the other hand, when 25 or 50 mM D-glucose was originally present, the decline in oxygen uptake rate at 230 min was not due to depletion of D-glucose.

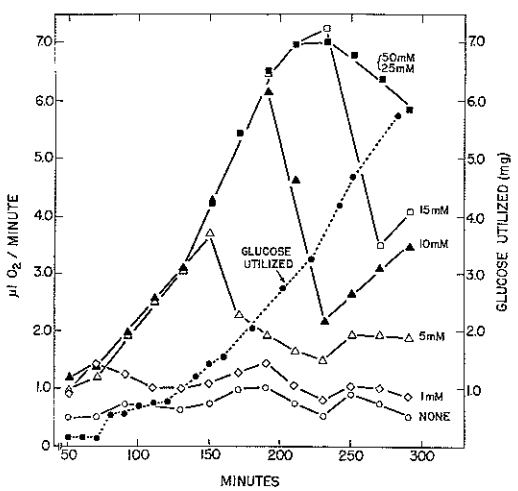

FIG. 7. *Oxygen uptake rates and utilization of glucose during postgerminative development of Bacillus megaterium spores. Spores were germinated for 30 min (80% germination) in* L-*alanine (25 mM), glucose (0.25 mM), K_2SO_4 (1 mM), and phosphate buffer (50 mM, pH 7.0) before the addition of various concentrations (1 to 50 mM) of glucose. Volume of reaction mixture was 1.5 ml. Oxygen consumption rates were plotted as solid lines; utilization of 25 mM glucose, as broken line.*

DISCUSSION

In many respects, this work with carbon compounds parallels our previous studies on nitrogenous compounds in germination and in postgerminative development of *B. megaterium* spores (Levinson and Hyatt, 1962; Hyatt and Levinson, 1962). In postgerminative development, carbon compounds are utilized for the synthesis of cellular material and as energy sources. Germination, on the other hand, may involve a "trigger" or activation mechanism, not necessarily using the germinant as a source of carbon. The 77 carbon compounds listed in Table 1 may be arranged into four groups, including all combinations of ability or inability to support germination or postgerminative development of *B. megaterium* spores. (i) Compounds which support both germination and postgerminative development are D-glucose, *N*-acetyl-D-glucosamine, D-fructose, β-methyl-D-glucoside, and D-glucosamine. (ii) Compounds which support germination but not postgerminative development are D-mannose, L-sorbose, D-allose, 2-deoxy-D-glucose, and 2-hydroxy-ethyl-D-glucose. (iii) Compounds which do not support germination but do support cell division are D-ribose, D-xylose, D-galactose, cellobiose, lactose, raffinose, maltose, sucrose, trehalose, turanose, melibiose, glycerol, mannitol, fumarate, malate, succinate, gluconate, 2-ketogluconate, L-aspartate, and L-glutamate; those which support elongation only are glucuronate, melezitose, citrate, inositol, and α-methyl-D-glucoside. (iv) Those compounds not supporting germination or postgerminative development are L-arabinose, pyruvate, lactate, L-rhamnose, acetate, sorbitol, D-glyceraldehyde, phosphorylated sugars, and 28 other compounds.

Many factors must be considered in determining whether a given compound is a germination agent. These include organism variables such as species or strain, spore population hetreogeneity, sporulation medium, and heat activation or aging of spores; substrate factors such as concentration, combinations of germinants, and ionic or other contamination; and physical factors such as pH and temperature of incubation.

The effectiveness of sugars as germination agents varies with the species or strain of *Bacillus* (Stedman, 1956). Thorley and Wolf (1961) re-

ported that, although none of their strains of *B. cereus* and *B. licheniformis* germinated on glucose, four of six strains of *B. megaterium* and two of three strains of *B. subtilis* did. Rode and Foster (1962*a*, *b*) distinguished between two types of *B. megaterium* strains, those which respond to glucose or mannose (our strain, QM B1551), and those, like *B. megaterium* Texas, whose germination is initiated best by a mixture of L-alanine and adenosine or inosine. Powell (1951) and Powell and Hunter (1958) demonstrated that heated spores of the glucose-mannose type of *B. megaterium* also germinated in glucosamine. None of the 11 sugars tested by Hermier (1962) at 100 mM gave more than 30% germination of heated (100 C, 30 min) *B. subtilis* spores, and the percentage of germination was not increased by the addition of amino acids. However, Hachisuka et al. (1955) found that, in the presence of L-asparagine (which did not, in itself, support germination), heated (80 C, 15 min) *B. subtilis* spores germinated at least 75% in caramelized glucose, maltose, fructose, lactose, or sucrose but, with the exception of maltose, not in the intact sugars. Halvorson and Church (1957) reported that freshly harvested *B. cereus* strain T spores, requiring both adenosine and heat activation, germinated rapidly in L-alanine but more slowly in glucose, pyruvate, acetate, or gluconate. Aging, however, relieved these spores of this requirement for adenosine and heat activation.

Because certain sugars such as D-glucose are effective germination agents at quite low concentrations, some caution should be used in interpreting the results when relatively high concentrations of other compounds, possibly contaminated with traces of glucose, are necessary for germination. For example, a number of sugars (e.g., D-galactose, α-methyl-D-glucoside, and gentiobiose) which promoted germination of heated spores, were devoid of germinative capacity when freed from glucose contamination. We cannot, on the basis of our observations on germination in reagent and in deionized sugars, agree with Rode and Foster (1962*a*) that contaminating ions bear a prime responsibility for germination in reagent glucose, although it is possible that we had particularly ion-free reagent sugars. Nevertheless, ions are important adjuvants, and germination in D-glucosamine·HCl is almost certainly due, in part, to Cl^-.

The compounds which we do consider to be active germination agents for our strain of *B. megaterium* include D-glucose, D-mannose, D-glucosamine, *N*-acetyl-D-glucosamine, 2-deoxy-D-glucose, 2-hydroxyethyl-D-glucose, D-allose, D-altrose, β-methyl-D-glucoside, D-fructose, and L-sorbose (Table 1). To the best of our knowledge, 2-deoxy-D-glucose and 2-hydroxyethyl-D-glucose have not previously been reported as germination agents for *Bacillus* spores. The effective compounds are all hexose sugars or their derivatives. D-Mannose, 2-deoxy-D-glucose, D-glucosamine, and *N*-acetyl-D-glucosamine, which support germination of unheated spores, differ from D-glucose only in the configuration at carbon atom 2. Compounds such as D-fructose, L-sorbose, β-methyl-D-glucoside, D-allose, and D-altrose, which are effective only with heated spores, differ from glucose configuration at additional carbon atoms (1, 3, or 5). Our evidence is inadequate to serve as a basis for speculation on the relationship between specific modifications of chemical structure and germination, as was done for amino acids by Woese, Morowitz, and Hutchison (1958).

We previously demonstrated, by variation of conditions for germination, that D-glucose and various nitrogenous compounds (notably L-alanine and L-valine) induce germination of *B. megaterium* spores by different metabolic pathways (Hyatt and Levinson, 1962). In the current parallel study, although some differences in the promotion of germination by five selected sugars are apparent, the evidence is not sufficient to warrant concluding that multiple pathways are operative in sugar-induced germination.

Postgerminative development of *B. megaterium* spores is supported by a wider variety of carbon compounds than is germination (Table 1). These carbon compounds include some pentoses and hexoses, some sugar derivatives, many disaccharides and oligosaccharides, some alcohols, and some of the more readily permeable tricarboxylic acid cycle intermediates. Phosphorylated sugars were neither oxidized nor used for postgerminative development. The rate and extent of development and the rate of oxygen consumption did not depend upon the class of compound, malate (a dicarboxylic acid), D-glucose (a monosaccharide), and trehalose (a disaccharide) all supporting equal development and similar oxygen uptake rates.

In our experience, glucose and other compounds are not oxidized by ungerminated spores,

but only postgerminatively (Hyatt and Levinson, 1962). In this we agree with Hachisuka and Sugai (1959) and with Goldman and Blumenthal (1964), but we have not observed glucose oxidation in the absence of germination, as was reported by Church and Halvorson (1957).

Our results are too fragmentary to justify any firm conclusions as to oxidative pathways during postgerminative development of *B. megaterium* spores. Glucose, gluconate, and fumarate utilization and oxidation are not inhibited by NaF, but are inhibited by Atebrin and by KCN. The lack of fluoride inhibition and the nonutilization of phosphorylated sugars suggest, but do not prove (with intact cells), that the Embden-Meyerhof (EM) pathway is inoperative during postgerminative development; inhibition by KCN (and utilization of malate, succinate, and fumarate) suggests developing functional terminal respiration and tricarboxylic acid cycle; Atebrin inhibition suggests a flavoprotein-mediated glucose-to-gluconate oxidation, perhaps by the hexose monophosphate (HMP) pathway. Hachisuka et al. (1956) noted an apparent change in the pathways for glucose oxidation as the germinated spore develops into a vegetative cell, glucose oxidation in the germinated *B. subtilis* spore being unaffected by fluoride or by cyanide (no EM pathway or tricarboxylic acid cycle), but vegetative forms being sensitive to these inhibitors. Contrary to the conclusion of Church and Halvorson (1957) that spores of *B. cereus* strain T lacked an operative EM pathway, Goldman and Blumenthal (1964) suggested a key role for the EM pathway at every stage of development, the relative importance of the HMP pathway decreasing after germination.

The precipitous drop in oxygen consumption rate when *B. megaterium* spores in 25 or 50 mM glucose (or in other carbon compounds) have undergone one cell division is not due to exhaustion of carbon source, but may reflect a shift in oxidative pathways, such as suggested recently by Goldman and Blumenthal (1964). Hachisuka, Asano, and Sugai (1958) noted a similar phenomenon, the glucose and gluconate oxidation rates of 4-hr cultured *B. subtilis* spores being higher than those of 6-hr cultured spores.

ACKNOWLEDGMENTS

We thank G. R. Mandels, M. Mandels, and E. T. Reese of the U.S. Army Natick Laboratories for critical reviews of the manuscript. F. W. Parrish of these laboratories purified certain sugars and performed paper chromatographic assays.

Samples of certain rare sugars were supplied by W. C. Boyd, Boston University Medical School; H. S. Isbell, National Bureau of Standards; and E. T. Reese and F. W. Parrish, U.S. Army Natick Laboratories.

LITERATURE CITED

CHURCH, B. D., AND H. HALVORSON. 1957. Intermediate metabolism of aerobic spores. I. Activation of glucose oxidation in spores of *Bacillus cereus var. terminalis*. J. Bacteriol. **73**:470–476.

GOLDMAN, M., AND H. J. BLUMENTHAL. 1964. Pathways of glucose catabolism in *Bacillus cereus*. J. Bacteriol. **87**:377–386.

HACHISUKA, Y., N. ASANO, M. KANEKO, AND T. KANBE. 1956. Evolution of respiratory enzyme system during germination of *Bacillus subtilis*. Science **124**:174–175.

HACHISUKA, Y., N. ASANO, AND K. SUGAI. 1958. Studies on spore germination. III. Development of enzymes concerning glucose oxidation during spore germination of *Bacillus subtilis*. Japan. J. Microbiol. **2**:79–88.

HACHISUKA, Y., N. KATO, N. ASANO, AND T. KUNO. 1955. Studies on spore germination. II. Effect of caramels from sugars and other carbon sources on spore germination. J. Bacteriol. **69**:407–412.

HACHISUKA, Y., AND K. SUGAI. 1959. Studies on spore germination. IV. Relationship between germination and appearance of glucose dehydrogenase activity in *B. subtilis* spore. Japan. J. Microbiol. **3**:211–222.

HALVORSON, H., AND B. D. CHURCH. 1957. Intermediate metabolism of aerobic spores. II. The relationship between oxidative metabolism and germination. J. Appl. Bacteriol. **20**:359–372.

HERMIER, J. 1962. La germination de la spore de *Bacillus subtilis*. I. Action des sucres et des acides amines sur la phase initiale de la germination. Ann. Inst. Pasteur **102**:629–643.

HILLS, G. M. 1950. Chemical factors in the germination of spore-bearing aerobes: observations on the influence of species, strain and conditions of growth. J. Gen. Microbiol. **4**:38–47.

HYATT, M. T., AND H. S. LEVINSON. 1957. Sulfur requirement for postgerminative development of *Bacillus megaterium* spores. J. Bacteriol. **74**:87–93.

HYATT, M. T., AND H. S. LEVINSON. 1959. Utilization of phosphates in the postgerminative development of spores of *Bacillus megaterium*. J. Bacteriol. **77**:487–496.

HYATT, M. T., AND H. S. LEVINSON. 1962. Conditions affecting *Bacillus megaterium* spore germination in glucose or various nitrogenous compounds. J. Bacteriol. **83**:1231–1237.

LEVINSON, H. S., AND M. T. HYATT. 1956. Correlation of respiratory activity with phases of spore germination and growth in *Bacillus megaterium* as influenced by manganese and L-alanine. J. Bacteriol. **72**:176–183.

LEVINSON, H. S., AND M. T. HYATT. 1962. Nitrogenous compounds in germination and postgerminative development of *Bacillus megaterium* spores. J. Bacteriol. **83**:1224–1230.

LEVINSON, H. S., AND M. T. HYATT. 1964. Effect of sporulation medium on heat resistance, chemical composition, and germination of *Bacillus megaterium* spores. J. Bacteriol. **87**: 876–886.

LEVINSON, H. S., AND M. G. SEVAG. 1953. Stimulation of germination and respiration of the spores of *Bacillus megatherium* by manganese and monovalent anions. J. Gen. Physiol. **36**:617–629.

PHILLIPS, J. D., AND A. POLLARD. 1953. Degradation of sugars on ion-exchange columns of "Amberlite IRA 400 (OH^-)." Nature **171**:41–42.

POWELL, J. F. 1951. The sporulation and germination of a strain of *Bacillus megatherium*. J. Gen. Microbiol. **5**:993–1000.

POWELL, J. F., AND J. R. HUNTER. 1958. The stimulation and inhibition of spore germination in *Bacillus* species. Intern. Congr. Microbiol. 7th, Stockholm, p. 43.

RODE, L. J., AND J. W. FOSTER. 1962*a*. Ionic germination of spores of *Bacillus megaterium* QM B1551. Arch. Mikrobiol. **43**:183–200.

RODE, L. J., AND J. W. FOSTER. 1962*b*. Ionic and nonionic compounds in the germination of spores of *Bacillus megaterium* Texas. Arch. Mikrobiol. **43**:201–212.

STEDMAN, R. L. 1956. Biochemical aspects of bacterial endospore formation and germination. Am. J. Pharm. **128**:84–97, 114–130.

SUMNER, J. B. 1925. A more specific reagent for the determination of sugar in urine. J. Biol. Chem. **65**:393–395.

THORLEY, C. M., AND J. WOLF. 1961. Some germination factors of mesophilic spore formers, p. 1–13. *In* H. O. Halvorson [ed.], Spores II. Burgess Publishing Co., Minneapolis.

WOESE, C. R., H. J. MOROWITZ, AND C. A. HUTCHISON III. 1958. Analysis of action of L-alanine analogues in spore germination. J. Bacteriol. **76**:578–588.